Probing pH-triggered self-assembling peptide based tumor imaging contrast agents in blood serum

Cancer is the second most common cause of death in the US.[1] According to the American Chemical Society, early diagnosis is the most effective way to treat cancer.[2] Although the advent of nanotechnology has greatly revolutionized the field of novel drug development for cancer treatment, early detection and therapy still remains a major challenge. Most drugs are designed to actively target receptors on the cancer-cell surface, but cancer cells mutate rapidly, altering receptor structure and allowing tumors to escape receptor-targeted drugs.[3,4] Another strategy extensively used is passive targeting exploiting the Enhanced Permeation and Retention (EPR) effect observed in most tumors.[5] The EPR effect occurs due to structural abnormalities (presence of holes and fenestrations) in the tumor vasculature resulting in selective leakage of nanoparticles into the tumor site compared to normal tissue.[6] However, this has shown mixed results too due to variations in tumor vasculature morphologies and abnormalities in fluid dynamics within tumors preventing accumulation of these drugs at the tumor site.[7]

Developing dynamically-triggered imaging pharmaceuticals that respond (structurally and/or functionally) to and preferentially localize at the tumor site via one of cancer's broader hallmarks rather than specific biomarkers can have a direct impact on decreasing the number of cancer related deaths. For cancer, one particularly attractive hallmark target is the acidic extracellular microenvironment of tumor tissue (pHe 6.6−7.4) that arises due to the enhanced rate of glycolysis found in most cancer cells.[8] Creating a material that is nano-sized in blood, but upon reaching the acidic extracellular tumor environment, transforms into a bulky, micro-sized, more slowly diffusing object could serve as a novel mechanism for achieving high accumulation

of imaging, drug delivery, or radiotherapeutic agents at the tumor site compared to the bloodstream. Especially for tumors, the heterogeneous matrix and high interstitial fluid pressure should govern the blood-tumor diffusion of nano- and micro-particles dependent on their size.[7]

To this effect, we have designed and synthesized bio-compatible self-assembling peptide amphiphile (PA) molecules that transform from– isolated molecules or spherical micelles into nanofibers (several microns in length) when the pH is slightly reduced from 7.4 to 6.6 via charge screening, in isotonic salt solutions that simulate the acidic extracellular microenvironment (pHe) of malignant tumor tissue **(Fig 1a)**.[9] Our PA molecules, in general, consist of four main segments: a hydrophobic palmitoyl tail, a β-sheet-forming peptide sequence, a charged amino acid (glutamic acid) sequence and a DO3A-Gd Magnetic Resonance imaging (MRI) moiety **(Fig 1b)**. The lanthanide ion can be replaced for various other applications. By balancing the hydrophobic attractive, electrostatic repulsive and steric forces, the self assembly morphology and pH of transition in these molecules can be systematically shifted. The PAs were synthesized via the solid phase technique using Fmoc chemistry, purified by Reverse-phase High Performance Liquid Chromatography (HPLC) and characterized by Mass Spectrometry, analytical HPLC and peptide content analyses.

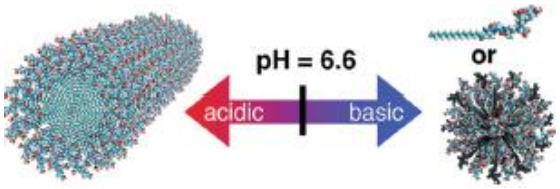


Fig 1a

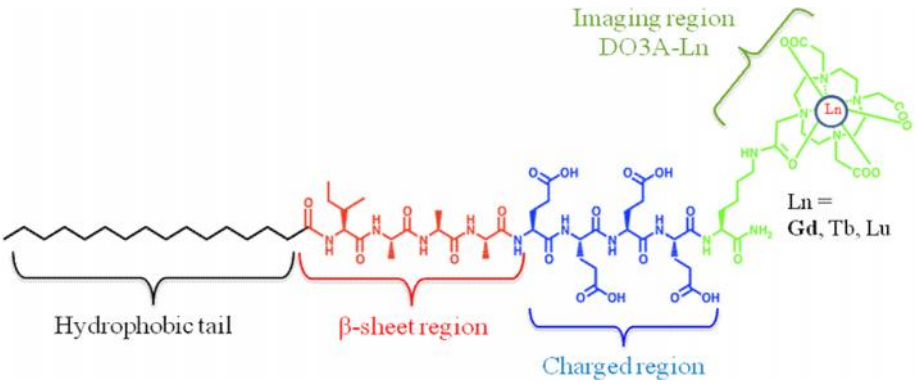


Fig 1b

Circular Dichroism (CD) spectroscopy, Critical Aggregation Concentration (CAC) measurements and conventional Transmission Electron Microscopy (TEM) using negative staining were used to characterize the pH dependent transition and self assembly structures in the PA molecules. CAC measurements via the pyrene 1:3 method[10] were used to ascertain single molecule to spherical micelle or nanofiber transitions at varying pH values ranging from 4.0-10.0. CD spectroscopy that probes peptide/protein secondary structure, was used to determine the pH points at which different concentrations (10-500 μM) of the PA molecules transitioned from a random coil to a β-sheet peptide structure. TEM showed that the random coil and β-sheet profiles correspond to spherical micelle and nanofiber morphologies respectively. After extensive evaluation of structure-property relationships in a series of PA molecules via systematically altering the hydrophobic and hydrophilic regions, the particular sequence Palmitoyl-IAAAEEEEK(DO3A:Gd) was found to transition at pH values closest to our desired physiological conditions. The transition points and images generated from CAC, CD and TEM were used to generate a concentration-pH phase diagram for this molecule **(Fig 2a)**.[9] All tests were done in 150 mM NaCl and 2.2 mM $CaCl_2$ to roughly mimic the ionic strength of blood,

since there is a strong dependence of self assembly of amphiphiles on salt concentrations. The transition was found to be rapid and reversible with respect to pH, indicating the system is under thermodynamic equilibrium. This was tested by switching the pH of a 100 µM PA solution back and forth between 6.0-6.10 and 7.70-7.75 followed by collection of CD spectra within 2-3 min of pH adjustment. The CD curves showed almost super imposable secondary structures for the acidic and basic pH values **(Fig 2b)** .[9]

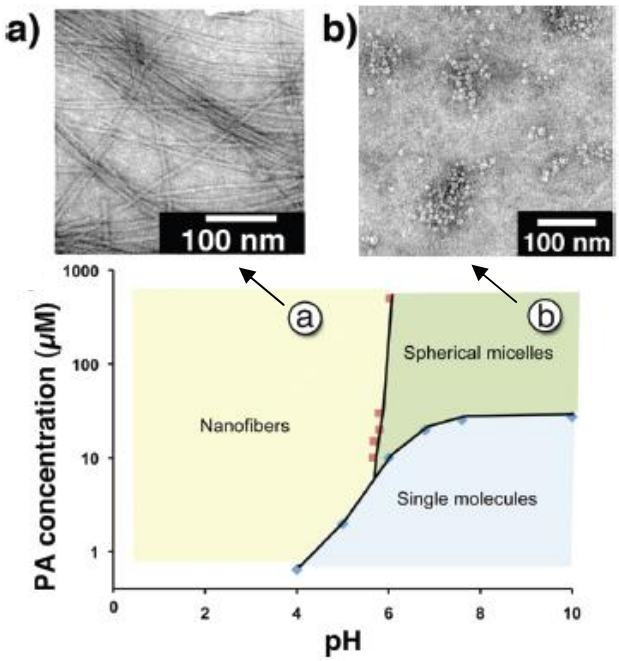


Fig 2a

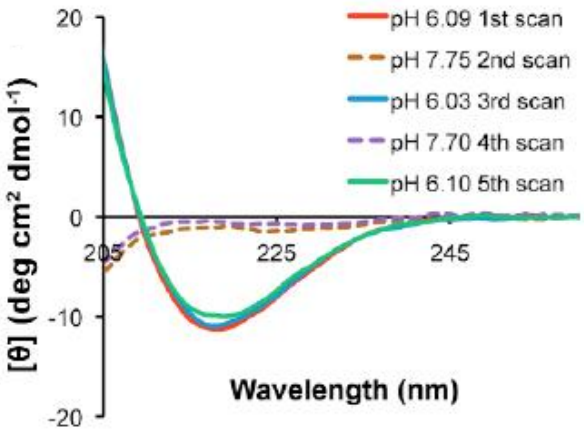


Fig 2b

Using MRI measurements with a 1.5T magnet, relaxivity values of water protons in the presence of the PA at pH of 4 (nanofibers) and 10 (spherical micelles), were found to be 8.3 and 6.6 mM^{-1} s^{-1}, respectively **(Fig 3).**[9] These values were higher than those measured for a Magnevist control standard (4.5 mM^{-1} s^{-1}) as expected. This relaxivity increase from nanofibers

to micelles is likely due to longer rotational correlation times when contrast agents are coupled to large molecular weight objects[11].

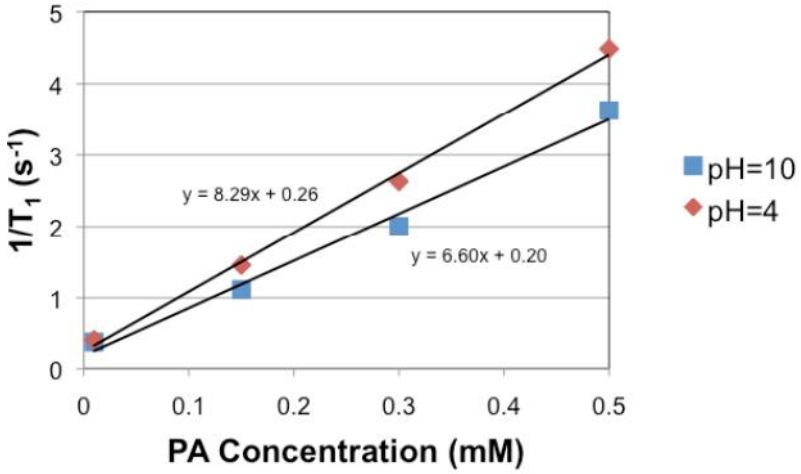
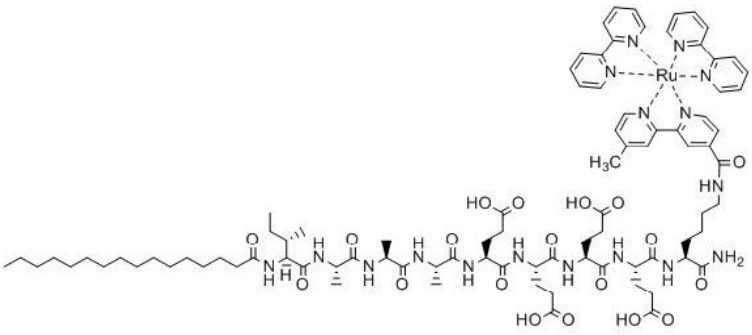


Fig 3

It is incredibly challenging to probe this peptide self-assembly behavior in a more realistic environment, for instance blood serum that contains numerous salts, proteins and enzymes. Fluorescence Anisotropy (FA) using a fluorescently-labeled PA was used for this purpose since CD or CAC cannot be used in presence of blood serum proteins. FA measures the extent of de-correlation of the polarized emission from a fluorescent dye with respect to the polarization of the excitation light, which linearly depends on the tumbling rate of the dye containing rotating unit in solution and consequently its size and molecular weight.[11] The dye chosen was Bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine ruthenium (II) and was conjugated to the lysine ε-amine group of the PA Palmitoyl-IAAAEEEEK-NH_2 **(**PA-RubiPy**, Fig 4a)**[12] via an NHS ester linkage. This dye has excitation and emission wavelengths of 450 and 630 nm respectively and a relatively high fluorescence lifetime of ~400-500 ns, appropriate for

FA measurements. The PA-RubiPy was spiked into our previously designed and characterized Palmitoyl-IAAAEEEEK(DO3A:Gd) in a small amount (1.5% of the total PA concentration) for pH dependent FA measurements. We found this mixed system to be under thermodynamic equilibrium (reversible w.r.t pH) and generate an appreciable pH independent fluorescence signal for FA determination **(Fig 4b)**.[12]



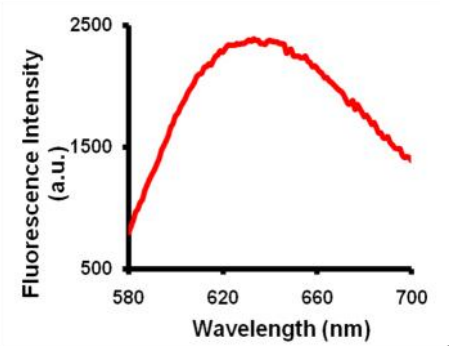


Fig 4a **Fig 4b**

Formation of bulkier, high molecular weight structures is indicated by an abrupt increase in FA due to increase in rotational correlation time of the rotating unit containing the dye. pH dependent FA values were obtained for a 100 μM PA mixture initially in our salt buffer and was found to correlate well with the CD spectra of the same. The FA values increased from 0.66 to 0.115 as the pH was lowered indicating formation of a bigger morphology. A transition onset pH of ~7.0 was confirmed from both the techniques.[12] However, FA measured in pure blood serum showed a constant value of 0.21 at all pH values, probably due to formation of fibers even at basic pH values due to interaction with proteins and excess salts in the serum. To elucidate the effect of blood serum on self-assembly behavior, pH dependent FA values were computed for the mixed PA system in solutions containing varying serum concentrations (0.75-4% v/v in 150

mM NaCl, 2.2 mM $CaCl_2$ buffer) **(Fig 5a)**.[12] The transition onset and endpoints shifted to more basic values, relative to that in the salt buffer (0%), upon addition of greater concentrations of serum, until no transition was observed in 4% serum. Similar to the values observed in the salt buffer, the FA increased from ~0.065 to ~0.12 as the pH was lowered. At 4% serum, the FA was found to be constant at ~0.125.FA values obtained for the corresponding serum background samples ranged from 0.003-0.005, indicating that there is no contribution from the serum auto-fluorescence. Controls containing just the PA-RubiPy in serum (Control 1) and the dye directly conjugated to pure mouse serum albumin (MSA, control 2) showed a constant FA value of 0.04-0.05 over pH and time, indicating that the FA jump is due to formation of a bulky morphology and not just the PA-RubiPy single molecules bound to serum albumin. TEM confirmed the morphologies at pH 6.85 (fibers) and pH 9.21 (indistinct globules) for the 1.5% serum sample **(Fig 5b).**[12]

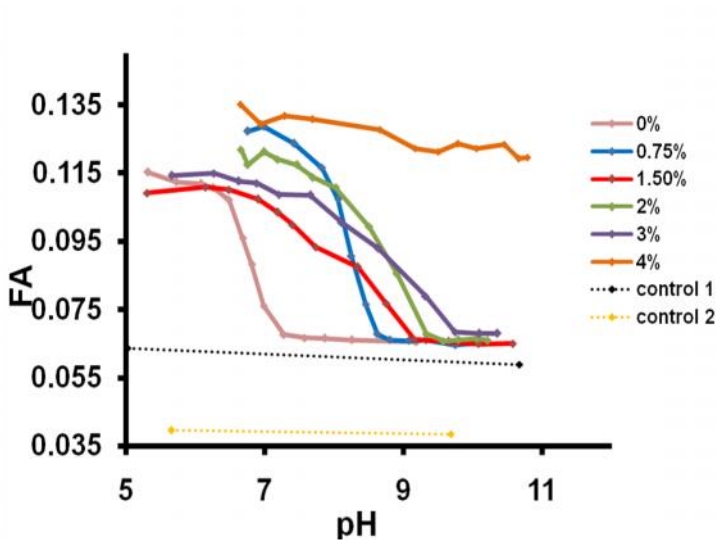


Fig 5a

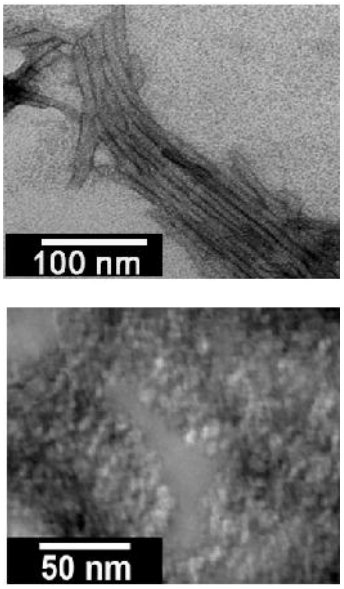


Fig 5b

The increased propensity for nanofiber formation in serum could be due to two probable factors: a higher ionic strength relative to our buffer and/or proteins, specifically albumin that typically constitutes ~75-80% (35-50 g/L) of all proteins in serum. Further control FA measurements in pure MSA indicated that albumin, surprisingly facilities this transition shift and favors fiber formation under basic conditions **(Fig 6)**.[12] The effect of salts was found to be minimal.

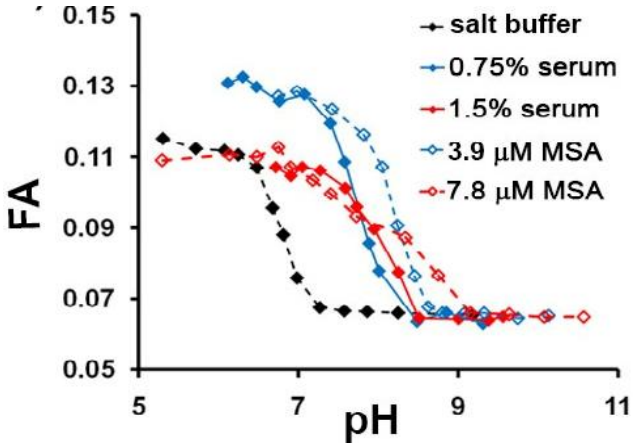


Fig 6

Although further structure optimization and *in vivo* experiments are in progress, we have demonstrated that via rational design, it is possible to create dynamic peptide amphiphile molecules that undergo self-assembly morphology transition in response to slight pH changes in salt concentrations similar to blood. These molecules were found to increase water relaxivity compared to a standard imaging agent control. We have also developed a method to probe this transition in blood serum using Fluorescence Anisotropy. The PA system was found to be under thermodynamic equilibrium which is an essential prerequisite for *in vivo* imaging and drug delivery applications.

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